The N-methyl-d-aspartate receptor antagonist CPP alters synapse and spine structure and impairs long-term potentiation and long-term depression induced morphological plasticity in dentate gyrus of the awake rat

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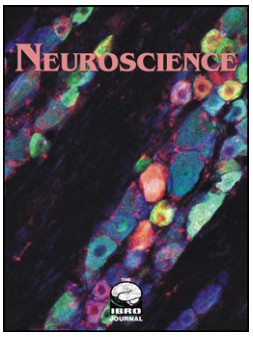

The NMDA Receptor Antagonist CPP Alters Synapse and Spine Structure and Impairs LTP and LTD Induced Morphological Plasticity in Dentate Gyrus of the Awake Rat

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THE NMDA RECEPTOR ANTAGONIST CPP ALTERS SYNAPSE AND SPINE STRUCTURE AND IMPAIRS LTP AND LTD INDUCED MORPHOLOGICAL PLASTICITY IN DENTATE GYRUS OF THE AWAKE RAT

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Running title: CPP impairment of LTP and LTD induced morphological plasticity

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List of abbreviations

LTP- long-term potentiation

LTD- long-term depression

CPP- (3-[(R)-2-Carboxypiperazin-4-yl]-propyl-1-phosphonic acid).

NMDA - N-methyl-D-aspartate

PSDs - postsynaptic densities

DG dentate gyrus

MML- middle of the molecular layer (of dentate gyrus)

MPP – medial perforant path

LPP - lateral perforant path

HFS - high-frequency stimulation

Abstract

Long-term morphological synaptic changes associated with homosynaptic long-term potentiation (LTP) and heterosynaptic long-term depression (LTD) *in vivo*, in awake adult rats were analysed using three dimensional (3-D) reconstructions of electron microscope images of ultrathin serial sections from the molecular layer of the dentate gyrus. For the first time in morphological studies, the specificity of the effects of LTP and LTD on both spine and synapse ultrastructure was determined using an NMDA receptor antagonist CPP (3-[(R)-2-Carboxypiperazin-4-yl]-propyl-1-phosphonic acid).

There were no differences in synaptic density 24h after LTP or LTD induction, and CPP alone had no effect on synaptic density. LTP increased significantly the proportion of mushroom spines, whereas LTD increased the proportion of thin spines, and both LTP and LTD decreased stubby spine number. Both LTP and LTD increased significantly spine head evaginations (spinules) into synaptic boutons and CPP blocked these changes. Synaptic boutons were smaller after LTD, indicating a pre-synaptic effect. Interestingly, CPP alone decreased bouton and mushroom spine volumes, as well as PSD volume of mushroom spines.

These data show similarities, but also some clear differences, between the effects of LTP and LTD on spine and synaptic morphology. Although CPP blocks both LTP and LTD, and impairs most morphological changes in spines and synapses, CPP alone was shown to exert effects on aspects of spine and synaptic structure.

Key Words: 3-D synaptic ultrastructure, hippocampus, long-term potentiation and depression

Long-term potentiation (LTP) and its counterpart long-term depression (LTD) are utilized widely as models of synaptic plasticity (Staubli and Lynch, 1987; Abraham et al., 1994; Doyère et al., 1997; Abraham, 2003; Malenka and Bear, 2004) that may be implicated in processes related to learning and memory storage (Doyère et al., 1993; Kemp and Manahan-Vaughan, 2007). Morphological studies have mainly focused on LTP within time ranges under 6h, few have characterized changes after one day. Moreover, the question of whether these LTP or LTD related long-term changes would be blocked by N-methyl-D-aspartate (NMDA) receptor antagonism has not yet been addressed.

Most morphological studies characterising morphological changes after LTD have been mainly in slices. A quantitative 2-dimensional ultrastructural study *in vivo* looked at morphological correlates of heterosynaptic LTD in dentate gyrus of the awake rat (Mezey et al., 2004) and showed that LTD was not simply the converse of LTP. A non input-specific increase was found in unperforated axospinous synapses 24h after both LTP and LTD while the major effect of LTD alone was an input-specific increase in axodendritic synapse density. The ideal way to examine morphological changes would be using combined 2-photon microscopy to visualise spines followed by electron microscopy to study the presynaptic boutons. Using such a technique in mouse organotypic hippocampal slice cultures, Becker et al. (2008) showed that structural plasticity following LTD is a feature that is not specific to dendritic spines, and presynaptic structural changes appeared to play a more important role than spine changes given that the gain or loss of contacts was frequently due to bouton changes. Bastrikova et al. (2008) used slices of rat hippocampus cultured on multielectrode arrays to demonstrate that LTD induction results in complete separation of the presynaptic bouton from the dendritic spine but no loss of spines. Spine formation following LTP was demonstrated using confocal microscopy by Engert and Bonhoeffer (1999) and De Roo et al. (2008) have shown that learning-related patterns of activity that induce long-term potentiation act as a selection mechanism for the stabilization and localization of spines. The relationship between LTP and LTD was examined by Zhou et al. (2004) who applied two-photon time-lapse imaging of dendritic spines in hippocampal slices from neonatal rats to show that the induction of homosynaptic LTD by low-frequency stimulation is associated with shrinkage of spines which was reversed by subsequent high-frequency stimulation that induces LTP. De Roo et al. (2008), in a confocal imaging study with organotypic slices, demonstrated that spine enlargement and spine stabilization following LTP were both NMDA receptor and protein synthesis-dependent.

However, two-photon microscopy cannot at present be applied *in vivo* to the hippocampus

from awake animals, and therefore precludes the observation of long-term changes associated with LTP or LTD *in vivo*. Furthermore, resolution limitations mean that it is difficult to determine fine details of changes in the pre-synaptic component of synapses, or the post-synaptic density (PSD). The present study therefore aimed to assess the long-term 3D morphological correlates of synaptic plasticity, 24 hours after induction of LTP and heterosynaptic LTD in middle molecular layer of the dentate gyrus of adult awake rats. The question of whether the effects of LTP and LTD on both spine and synapse ultrastructure changes are specific to potentiation or depression *per se,* and therefore also prevented by blockade of NMDA, was determined by use of the NMDA competitive receptor antagonist, CPP (3-[(R)-2-Carboxypiperazin-4-yl]-propyl-1-phosphonic acid).

Materials and Methods

Animals Eighteen adult male Sprague-Dawley rats (Iffa Credo, France), ~8-10 weeks old and weighing 300- 350 g at the time of surgery, were used as subjects. They were housed individually with food and water *ad libitum* in a temperature-controlled room and on a 12-h light/dark cycle. All animal experimental procedures were carried out at Neurobiologie de l'Apprentissage, de la Mémoire et de la Communication, CNRS-Université Paris-Sud, in accordance with guidelines of the EU, CNRS, and the French Agricultural and Forestry Ministry (decree 87848; licence no. A91429). Animals were anaesthetized with sodium pentobarbitone (60 mg/kg i.p.) and prepared for chronic recording as described previously (Doyère et al., 1997; Mezey et al., 2004). Two 65-μm nichrome recording wires extending 1.5 mm from a stainless steel microtube were implanted in the hilus of the left dentate gyrus (AP 4.2 mm; L 2.5 mm from Bregma) and two concentric bipolar electrodes ipsilaterally to activate the lateral perforant path (AP 8.2 mm, L 4.8/5.2 mm from Bregma), and the medial perforant path (AP 7.8 mm, L 4.2 mm from Bregma). The depths of the recording and stimulating electrodes were adjusted to maximize the slope of the positive-going field excitatory postsynaptic potential (EPSP) evoked by stimulation of the pathway. The microtube served as a reference and a cortical silver ball served as a ground. All electrodes were connected to multichannel miniature sockets, fixed to the skull with dental acrylic. Rats received a daily injection of antibiotic (Terramycin, 20 mg/kg, i.p.) for 5 days and were allowed to recover from surgery in their home cages for at least 10 days.

Induction of long-term potentiation/long-term depression Experimental procedures were similar to those previously reported (Mezey et al., 2004). In short, after 3 days of habituation to the recording chamber (30 min each day) during which input/output curves and classical summation and convergence tests were performed, animals were submitted to a three-day experimental design: For baseline, field potentials were evoked alternately on the two pathways at 15s intervals for 20 min using test stimuli (80 μs) to evoke EPSP slopes of approximately half its maximum. On the day of LTP/LTD induction, after the baseline was taken, tetanic stimulation was delivered, and recordings were resumed for 30 min after the last tetanus. Tests were again taken 24h later, followed immediately by sacrifice of the animals. Hippocampal EEG was continuously monitored during recording sessions to ensure the lack of electrically-induced after discharges.

Experimental Groups

There were six animal groups for this ultrastructural study with all tissue taken from the middle of the molecular layer (MML) of the dentate gyrus (DG).

LTP group: LTP in middle molecular layer of DG - A strong tetanization protocol (ten series of seven 400-Hz trains, 1 s intertrain interval, 1 min between each series) was applied to the medial perforant path at test intensity.

LTP blocked by CPP group: Blockade of LTP by NMDA antagonist - Rats received an injection (10 mg/kg, i.p.) of the NMDA-receptor antagonist CPP (Tocris Cookson Ltd) 3 h before tetanization of the medial perforant path.

LTD group: LTD in middle molecular layer of DG – The tetanization protocol (above) was applied to the lateral perforant path.

LTD blocked by CPP group: LTD was blocked by CPP in the same way as in the LTP blocked by CPP group.

Control group with CPP (to test effect of CPP alone): Rats (n=3) received an injection (10 mg/kg, i.p.) of CPP as above but received no stimulation.

Control group: Rats (n=3) were either implanted, but received no stimulation (n=1), or received only single test stimulations over 4 days (n=2).

Perfusion fixation and microscopy Immediately after the last test stimulation or 28h after CPP injection in the control group, rats were anaesthetized with sodium pentobarbital (0.1 ml/100 g body weight) and perfused trans-cardially with 50 ml of 3.8% acrolein (TAAB, UK) in a solution of 2% paraformaldehyde and 0.1M phosphate buffer (PB), pH 7.4, followed by 250 ml of 2% paraformaldehyde. Brains were removed from the cranium and

cut into 4–5-mm coronal slabs of tissue containing the entire rostrocaudal extent of the hippocampus. The region containing the recording electrode was easily identified by the electrode tract markings. This tissue was further fixed for 30 min in 2% paraformaldehyde and then sectioned at 40–50 μm on a vibrating microtome (VT1000; Leica, Milton Keynes, UK). Four of these slices were taken from each animal 1-2 mm on either side of the electrode tract in the tetanized hemisphere. The tissue was osmicated in 2% osmium tetroxide and transferred to the Open University (Milton Keynes, UK). Further processing and embedding protocols were essentially similar to those reported previously (Stewart et al., 2005a, 2005b). Tissue was dehydrated in graded aqueous solutions of ethanol from 40 to 96% (each for 10 min) and then 100% acetone (three changes, each for 10 min). Specimens were infiltrated with a mixture of 50% epoxy resin and 50% pure acetone for 30 min at room temperature. Each slice was placed on an Aclar film and covered with a capsule containing pure epoxy resin (Epon 812 / Araldite M epoxy resins) for 1 h at 60 °C and polymerized overnight at 80 °C.

Slices in blocks were then coded and all further analyses were carried out with the investigator blind to the experimental status of the tissue. The embedded slices on the block surface were trimmed with a glass knife along the entire surface of the hippocampal slice and 1μm thick sections cut. A trapezoid area was prepared with a glass knife, with one side of 200–250 μm in length, which included the DG and a portion of hilus. This procedure is illustrated in Popov et al. (2004, 2005). Serial sections of grey / white colour (60–70 nm) were cut with a Diatome diamond knife and allowed to form a ribbon on the surface of a water / ethanol solution (2–5% ethanol in water) in the knife bath and collected using Pioloform-coated slot copper grids. Sections were counterstained with saturated ethanolic uranyl acetate, followed by lead citrate, and were then placed in a rotating grid holder to allow uniform orientation of sections on adjacent grids in the electron microscope.

Stereology of synapses Synapses in the MML is very largely those with an asymmetric synaptic junction and spherical type vesicles (i.e. Gray type I and presumed excitatory). Stereological estimates of synapse density in the MML of the DG were made 80–100 μm from the proximal edge of the granule cell body layer, the regions analysed being subject, as described above, to LTP or LTD induction by tetanization of the medial or lateral perforant path (MPP or LPP), respectively. Stereological analysis was performed as described previously (Harris, 1994; Fiala et al., 1998; Sorra and Harris, 1998, Popov et al., 2004), with

tissue volumes of approximately 500–800μm^3. The synaptic number was counted within these areas irrespective of the presence of components such as large dendrites and non-spiny dendrites of interneurons, in order to avoid bias in the data obtained. Synaptic densities were expressed as number of synapses (identified via postsynaptic densities (PSDs) and the presence of at least two presynaptic vesicles) per 100 μm^3 of tissue.

Density and proportion of different categories of spines and synapses Spines were categorised according to Peters and Kaiserman-Abramof (1970) and Harris et al. (1992), a process which is in agreement with an analysis of a histological preparation of Cajal's material by Garcıa-Lopez et al. (2006) and we have used this categorisation previously, (Popov et al., 2004). We thus distinguished 3 spine categories as shown in the 3-D reconstruction of a short segment of dendrite in Fig.1A, taken from over 100 serial ultrathin sections, 8 of which are shown in Fig. 1B with individual elements of types of spines labelled. A schematic of the spine categories is shown in Fig. 1C and can be compared with the reconstructions in Fig. 1A. The categories are thin- where the height is usually several times in excess of the width; stubby- where the spine protrudes only slightly from the dendritic shaft; mushroom- where the spine head is large and considerably in excess of the spine neck diameter, Shaft synapses are a fourth category where the synapse contacts directly the dendritic shaft (Figure 1A - C).

Digital reconstructive analysis Electron micrographs at x6000 magnification were obtained in a JEOL 1010 electron microscope from the MML at a distance of 80–100 μm from the layer of neuronal cell bodies. Up to 100 serial sections per series were photographed to reconstruct individual apical dendritic segments and their thin and mushroom spines and presynaptic boutons. A minimum of 25 thin and 25 mushroom spines, and 25 presynaptic boutons, were reconstructed per series obtained for each animal. As with the selection of animals, all series were also coded and analysed blind as to treatment condition, and all spines were chosen randomly. Identified cross-sectioned myelinated axons, mitochondria and dendrites spanning each section provided fiduciary references for initial alignment of serial sections. Section thickness was determined using the approach of Fiala and Harris (2001) and was normally 60–70 nm (grey ⁄ white colour). Digitally scanned electron microscopy negatives with a resolution of 1200 dpi were aligned as JPEG images (software available from http://synapses.bu.edu). Alignments were made with full-field images. When

section alignment had been finalised, the contours of individual dendritic spines, PSDs, axons, and mitochondria were traced digitally, and volumes, areas and total numbers of structures were computed. 3D reconstructions were exported to 3D-Studio-Max 8 software for rendering and subsequent rotation to display the optimal views of the reconstructed structures. Selected image files were finally imported into Adobe Photoshop CS2®, optimised for contrast and brightness, and montaged to produce illustrative figures for publication.

Statistical analyses For analyses of 3D measurements ANOVA tests were used to examine differences between specific animal groups followed by post-hoc comparisons, where required, using the Tukey's unequal N honest significant differences tests (implemented through Origin Pro 7.5). Formal statistical significance was taken where $P < 0.05$. Data are presented as mean ± SEM (n=3 animals per group).

Results

Induction of homosynaptic LTP and heterosynaptic LTD

There were four experimental groups (see Fig. 2) and two control groups for this study with all tissue taken from the middle of the molecular layer (MML) of the dentate gyrus (DG) for 3D reconstruction and morphological analysis:

(1) Animals for which the HFS protocol was applied to the medial perforant path to induce LTP at the level of the DG middle molecular layer (selected rats n=3) showed an immediate increase in both EPSP and population spike amplitude, an increase which remained 24h later, immediately before the sacrifice (Fig. 2A,B). Significant LTP of the EPSP slope was observed both 30 min ($t(2)=4.63$; $P<0.05$) and 24h after tetanization ($t(2)=9.50$; $P<0.05$).

(2) Animals for which LTP induction was blocked by the injection (10 mg/kg, i.p.) of the NMDA-receptor antagonist CPP 3 hours before HFS of the medial perforant path (selected rats n=3) showed a complete blockade of LTP for both EPSP and spike components (Fig. 2A,B). No significant change from baseline was observed 30 min after tetanisation ($t(2)=-0.41$; ns) or 24h later ($t(2)=2.34$; ns)

(3) Animals for which heterosynaptic LTD was induced in MML of DG by applying the tetanization protocol to the lateral perforant path (selected rats n=3) showed a significant LTD of EPSP slope, both 30 min ($t(2)=-4.38$; $P<0.05$) and 24h ($t(2)=-7.17$; $P<0.05$) after the tetanization (Fig. 2C,D).

(4) Animals for which heterosynaptic LTD was blocked by the injection (10 mg/kg, i.p.) of the NMDA-receptor antagonist CPP 3 hours before HFS of the lateral perforant path (selected rats n=3) showed a complete blockade of LTD for both EPSP and spike components (Fig. 2C,D), confirming NMDA-dependence of heterosynaptic LTD (Christie and Abraham, 1992; Kosub et al., 2005).

(5) Control group with CPP*:* Rats (n=3) received an injection (10 mg/kg, i.p.) of CPP as above but received no stimulation.

(6) Controls rats (n=3) for which no HFS was applied. No changes in EPSP were recorded.

Synapse density Two electron micrographs (from a series of 100) containing identified synapses and spines are shown in Fig. 3 (a, b). Reconstructions of 100 serial sections of the synapse shown in (a) and (b), and presented in Fig. 3c. This example is of a synaptic contact on a mushroom spine and 2 spinules from this mushroom spine arise from discontinuities in the PSD on the spine head and evaginate into the pre-synaptic bouton. Fig. (3d,e) are 2 serial electron micrographs from a thin spine in which 2 spinules project into an axon, and these are seen clearly in the 3-D reconstruction in Fig. 3(e). Spinules from thin spine heads are less frequent than on mushroom spines (see below). Mean synapse density per 100 μm^3 was 298±12 in control (there was no significant difference in synapse density between controls with and without CPP), 287±4 in LTP, 286±14 in LTD, 304±11 in CPP-LTP treated rats and 291±5 in CPP-LTD rats. There were no significant differences in these synapse density values between the 6 groups after either *in vivo* induction of LTP or LTD in awake rats, or in rats where LTP or LTD was blocked by CPP. These data show clearly that neither LTP nor LTD affects synapse density *in vivo,* at least at the 24h time point after their induction.

Synapse/spine types A schematic diagram showing features typical of spine synapses in the MML was presented in Fig. 1. The 3 axo-spinous categories examined were: mushroom, thin, stubby, plus an additional category (shaft) where synapses make contact directly on the dendritic shaft (axo-dendritic). Approximately 250 synaptic contacts on spines/shaft were analysed per animal for these measurement, and the proportions of each of the 4 categories from this number are presented in Fig. 4A. There was a large and statistically significant increase in the proportion of mushroom spines (~50%) in the MML after LTP induction compared to control and LTD induction ($F_{1, 8}$ =7.4, $p<0.05$) an effect which was blocked by

CPP. There was a small but significant increase following LTD in the proportion of thin spines (~8% compared to control), which was blocked by CPP. No change in the proportion of thin spines was observed after LTP treatment. When the change in proportion of thin spines after LTD was compared to that after LTP the difference was highly significant ($F_{1,8}$ =12.74, p<0.02). Both LTP and LTD decreased significantly the proportion of stubby spines (Fig. 4A) (LTP vs. control $F_{1,8}$ =13.12, p<0.005); LTD vs. control $F_{1,8}$ =7.51, p< 0.03); CPP blocked these changes. There were no changes in spine proportions from application of CPP alone. There were no significant changes in the proportions of shaft (axo-dendritic) synapses in any of the 4 experimental groups.

Spinules Spinules are suggested to play a role in exchange of material at synaptic junctions and may also affect the electrical coupling of the pre/post-synaptic structures. In light of these hypotheses we asked whether spinules are differentially present following LTP or LTD and this was assessed quantitatively. In Fig. 4B, it can be seen that the number of spinules per spine increased significantly on mushroom spines in the MML from 0.2 per spine in control to 1.4 after induction of LTP ($F_{1,8}$ = 106, p<0.001), and 0.98 after LTD ($F_{1,8}$ = 58.8, p<0.001); these increases were completely blocked by CPP. Increases of similar scale and significance in spinule number on thin spines were found after both LTP ($F_{1,8}$ = 16.3, p<0.001) and LTD ($F_{1,8}$ = 15.99, p<0.001 (from 0.09 per spine in control, to ~0.4 per spine after LTP or LTD)) and these too were blocked by CPP (Fig. 4B). CPP alone had no effect on spinule number of thin or mushroom spines.

Volume of mushroom and thin spines; volume of PSDs. Since the spines and PSDs were reconstructed from serial sections their volume could be calculated simply from measurement of the area of spine (or PSD) times the number of sections in which the structure occurred. From each animal the volume of 25 mushroom and 25 thin spines was measured from the 3-D reconstructions. The mean volume of mushroom and thin spines (~0.27 μm^3) did not change significantly after LTP or LTD (Fig. 5A,B). There was a significant reduction in volume to 0.22 μm^3 for mushroom spines when induction of LTP was preceded with CPP treatment ($F_{1,8}$= 8.9 p<0.04), and CPP also reduced the volume of mushroom spines when given to control animals ($F_{1,8}$=17.8, p<0.01). The volume of thin spines was not significantly affected by CPP alone, or when CPP treatment preceded LTP or LTD.

The volume of the PSDs of mushroom or thin spines was not significantly affected by LTP or LTD (Fig. 5C,D). However, CPP on its own caused a significant reduction in the volume of the PSDs on mushroom spines (Fig.5C, $F_{1,8}$=46.67, $p<0.002$). Mushroom spine PSD volume was ~20% lower when LTP was preceded by CPP treatment (Fig. 5C), as it was when CPP preceded LTD treatment but these reductions at $p<0.06$ were just below the significance criterion (Fig. 5C).

Presynaptic bouton volume (Fig. 6A, B). In order to determine whether LTP and LTD exerted an effect on presynaptic morphology, the volume of presynaptic boutons within a given area was determined by reconstruction of every presynaptic varicosity (presynaptic bouton) contacting mushroom spines to determine their volume, and the area of the apposition zone was also determined. An example of a reconstructed presynaptic bouton contacting 2 mushroom spines is shown in Figure 6A. Data for volume changes are shown in Figure 6B. Although the volume of presynaptic boutons was unaffected by LTP, it decreased significantly from ~0.6 μm^3 in the control group to ~0.35 μm^3 in both CPP ($F_{1,8}$ = 14.3, $p<0.02$) and LTP plus CPP group ($F_{1,8}$ = 49.4, $p<0.002$), LTD alone caused a significant reduction in volume of the presynaptic bouton ($F_{1,8}$ = 11.2, $p<0.03$)

The key findings of these experiments are summarized in table 1. They show that although most of the significant LTP- and LTD-induced changes were prevented by CPP; CPP alone applied to untetanised tissue also produced some long-lasting morphological changes in spines and synapses.

Discussion

In the search for correlates of the long-term stable state of plasticity, this study has examined morphological changes in dendritic spines and synapses in the middle molecular layer (MML) of the dentate gyrus of conscious adult rats 24h after LTP induction, and for the first time in ultrastructural studies, their specificity was examined when blocked by an NMDA receptor antagonist, CPP, or when compared with heterosynaptic LTD. These data are discussed in terms of the specificity of the various changes.

LTP-specific changes blocked by the NMDA antagonist CPP Changes in proportions of the different spine types after LTP – i.e. the increases in mushroom spines and the decrease in

stubby spines, were blocked by CPP. Similarly, the increase in number of spinules was blocked by CPP. All these changes blocked by CPP are most likely those induced by the physiological stimulation that leads to NMDA-dependent LTP. Therefore this dendritic spine remodelling, similar to that observed previously at earlier times post LTP (Kirov and Harris, 1999; Toni et al., 1999, 2001; Yuste and Bonhoeffer, 2001; Popov et al., 2004; Stewart et al., 2005b), *can* be considered as reflecting post-synaptic correlates of LTP. In addition, the present study demonstrates that these changes are present 24h after stimulation, and may therefore constitute correlates of a long-term maintenance phase of LTP. We cannot rule out the possibility that the dosage of CPP, although electrophysiologically potent, was insufficient to block all the changes associated with LTP, nor can we totally exclude a non-plasticity effect due to the tetanus, present in LTP and CPP groups but not the controls.

LTD-specific changes Correlates of LTD were also observed in the present study. As in our earlier 2-dimensional analyses (Mezey et al., 2004), morphologically the ultrastructural changes following heterosynaptic LTD are not simply a mirror of LTP. In contrast to LTP, LTD was accompanied by a specific increase in the number of thin spines and a decrease in the number of stubby spines. More importantly, there was a net decrease in bouton volume contacting mushroom spines. This latter result clearly shows a presynaptic correlate of heterosynaptic LTD in the dentate gyrus, which is supported by the 2-photon organotypic slice studies of Becker et al. (2008) from CA1 of mouse hippocampus where homosynaptic LTD induction increased the turnover of presynaptic boutons. Our findings are also interesting to consider in view of the study of Wang et al. (2007) on LTD in acute hippocampal slices of rat pups. Spine size was monitored and synaptic responses recorded simultaneously using combined two-photon time-lapse imaging with patch-clamp recording. They showed that spine shrinkage and LTD could occur independently of each other. Moreover they demonstrated that changes in spine size are unrelated to trafficking of AMPA receptors (AMPARs). There are, however, significant differences between our study and that of Wang et al. (2007); the latter was done in acute slices of very young rats, used homosynaptic LTD and was carried out at 2-photon level and did not therefore examine detailed ultrastructure in 3D. Whether or not homosynaptic and heterosynaptic LTD may differ in terms of their consequences at the morphological level remains to be clarified. However, it is pertinent to note the recent suggestion that ‘heterosynaptic’ LTD may require homosynaptic activity, and therefore be of ‘homosynaptic’ nature (Abraham et al., 2007), which would also be supported by the convergence of our data with Becker et al. (2008).

Correlates of LTP and LTD in spines and synapses Both LTP and LTD are hypothesized to participate in learning and memory processes (Doyère et al., 1993; Kemp and Manahan-Vaughan, 2007). It is therefore important to characterise carefully the changes observed after LTP and LTD, to allow the recognition of any of these changes that may occur after learning in a natural situation. There were no changes in synapse density after LTP or LTD in the MML 24h after initial stimulation, but we cannot rule out the possibility that such changes occur at earlier time points as part of a synaptic remodelling process and are not apparent at 24h post LTP induction. However, our data are in agreement with those from a number of previous studies (e.g see review by Geinisman, 2000), which showed that induction and maintenance phases of LTP are not associated with significant changes in the total number of synapses per postsynaptic neuron. Here, the most notable change in spine types involved an increase in the proportion of mushroom spines after LTP coupled with a decrease in stubby spines, while the proportion of thin spines remained stable. Large spines of the mushroom type, which invariably contain a spine apparatus, are believed to be the more stable type of spine and increase in number with age, and have therefore been termed "learning spines" by Bourne and Harris (2007), whereas thin spines are not stable in number (Holtmat et al., 2005). Large spines possess an abundance of smooth endoplasmic reticulum and polyribosomes (Spacek and Harris, 1997; Ostroff et al., 2002), which provide the synthetic machinery necessary for structural changes. Spines also contain a multitude of molecular components (Bourne and Harris, 2008) which may be influenced by experiential factors. De Roo et al. (2008) demonstrated in organotypic hippocampal slices that spine enlargement and stabilization following LTP were both NMDA and protein synthesis–dependent, which enables synaptic remodelling. The effect of stimulation on large spines most probably involves a sequence of intracellular biochemical events that includes changes in protein synthesis machinery and in cytoskeletal spine structure due to actin polymerization (Matus, 2000). This leads to an increase in spine size coupled to increased expression and insertion of AMPAR into the PSDs at the spine head (Ganeshina, 2004a, 2004b), which in turn would produce larger postsynaptic currents and hence participate in the expression of LTP (Matus, 2005). We have previously shown increases in the proportion of mushroom spines after chemical potentiation in CA1 in slices (Stewart et al., 2005a), after LTP induction in the dentate gyrus in anaesthetised rats (Popov et al., 2004), and after treatment of ageing rats with a neural cell adhesion molecule (NCAM) mimetic (Popov et al., 2008). Here, we show that this increase in mushroom spines in the hippocampus is

observable after LTP in awake adult rats, and is maintained for at least 24h, suggesting this is a correlate of the long-term maintenance of LTP. The present study also shows that, at a similar time in the same animals, there were no changes in thin spine proportions after LTP, but a decrease in stubby spines. In fact, the categories of spines we used most likely represent a continuum where stubby spines become thin and then become thick (mushroom) following neural activation, and *vice versa* when activity/down-regulation occurs. Therefore, we suggest that the changes observed after LTP most likely result from an effective equilibrium process, due to the transformation of the thin spines to mushroom in response to LTP, while the stubby types transform into the thin category. Following the same logic, in LTD the stubby spines would decrease in number as they form thin spines.

Correlates of LTP and LTD in changes in spinule number Spinule number increased after LTP. Spinules, which are double-membrane structures (as found by Spacek and Harris, 2004), were observed to emerge from both mushroom and thin spine heads, and penetrate for considerable distances into the presynaptic terminals. Spacek and Harris (2004) noted that spinules engulfed by astrocytic processes support the growing evidence that perisynaptic glia interact directly with synapses at least on thin spines. The observation of spinules emerging from thin spines contrasts that of a recent study by Nicholson and Geinisman (2009), which found that neither spinules nor spine apparati were found in typical non perforated synapses, which we take to be the equivalent of the thin spines we observed. While we agree that spine apparati are never present in thin spines we do consistently find spinules on thin spines in our study, albeit at lower levels than in mushroom spines. However, there are some differences between the two studies, while we examined dentate middle molecular layer, that of Nicholson and Geinisman (2009) examined CA1 synapses in non-tetanised tissue.

Toni et al. (2001) showed that 30 min after LTP induction in hippocampal organotypic slice cultures from neonatal rats, there was an increase in segmented synapses and an increase in large spinules associated with PSD. Tao-Cheng et al., (2009) demonstrated that while spinules in hippocampal slice cultures were virtually absent in control slices, numerous spinules appeared at both excitatory and inhibitory synapses after treatment with depolarisation induced by high K, and to a lesser extent following NMDA treatment. The authors suggested that rapid turnover of synaptic spinules represents an aspect of membrane retrieval during synaptic activity. Earlier studies had also suggested that spinules were involved in remodelling the postsynaptic membrane, as indicated by their transient increase

shortly after LTP induction (Applegate and Landfield, 1988; Schuster et al., 1990; Geinisman et al., 1993; Toni et al., 1999), though here they were present 24h after initial tetanisation. The function of the spinules is unclear, but may be related to growth and remodelling of synaptic membranes or an increased motility of spines (Matus, 2000). Spacek and Harris (2004) considered several possibilities for the role of spinules. Most relevant to the present study is the possibility that trans-endocytosis of spinules removes excess pre- and postsynaptic membrane after activation resulting in transient or segmented synapses on mushroom spines. Spinules could also serve to affect the electro-morphological geometry of the pre- and postsynaptic elements to strategically affect their electrical coupling and performance. Nevertheless the clear evidence from stimulation in the present study – whether LTP or LTD inducing, suggests that remodelling of the postsynaptic membrane occurs with long-term plasticity, which is supported by the fact that CPP completely blocks the increases in spinules.

Changes induced by CPP The present data highlight for the first time the long-term effect of a single i.p. injection of an NMDA antagonist CPP, on synapse and spine ultrastructure in the hippocampus. Indeed, CPP not only blocked most of the LTP and LTD related changes, but also whether acting in combination with tetanisation, or alone, induced other changes. These are an overall decrease in spine volume for mushroom but not thin types, as well as a decrease in the volume of presynaptic boutons contacting mushroom spines, and a decrease in the volume of PSD on mushroom spines. The latter result suggests that the NMDA antagonist alone possibly induces some receptor removal since it can induce this effect even without any tetanisation. Increases in the size of the spine head or PSD have been suggested to be due to the intensive processes correlated with AMPA receptor insertion and receptor recycling (Ganeshina et al. 2004a, 2004b; Nicholson et al. 2004, 2006). However, the absence of such changes here and the fact that CPP actually caused a decrease in PSD volume suggests that any process of receptor insertion either has not occurred or occurs outside the timeframe of the experiments in our study, or indeed, as with the change in spine volume, reflects the fact that CPP actually removes receptors. It seems reasonable to suggest that changes associated with CPP are not necessarily restricted to the dentate gyrus and that they may be the basis for the durable behavioural effects that NMDA antagonists have been reported to produce when injected acutely (Manahan-Vaughan et al., 2008).

In summary, our data show that there are a number of clear similarities, but also some distinct differences, between the effects of LTP and LTD on spine and synaptic morphology *in vivo* in awake animals. Furthermore, when CPP blocks LTP and LTD, it impairs most, of the ultrastructural changes. We suggest that these changes in synapse and spine structure are therefore a morphological correlate, a *de facto* memory, of neurophysiological activity linked to LTP and LTD. How these modifications compare with those induced in natural conditions during memory formation is a question to be addressed in further studies.

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TABLE 1. Summary of the major morphological changes in synapses and dendritic spines in the middle molecular of the dentate gyrus 24h after induction of LTP or LTD in awake adult rats, in comparison to untetanised but implanted controls. Synapse density and spine proportions were determined from hippocampal tissue volumes of each animal of between 500-800μm^3. All analyses of spinules numbers and spine volumes were made from 3 -D reconstructions of ultrathin serial sections viewed in an electron microscope from 25 of each spine type, and from 25 boutons on mushroom spines, from each of 3 animals per treatment groups. The effect of blockade of LTP and LTD by CPP is also reported. Effects recorded as increased or decreased are significant at $p<0.05$.

STRUCTURAL PARAMETER	**LTP**	**CPP (effect on change in LTP)**	**LTD**	**CPP (effect on change in LTD)**	**CPP alone**
Synapse Density	No change	No change	No change	No change	No change
Spine Number:					
(i) Mushroom	Increase	Blocked	No change	No change	No change
(ii) Thin	No change	No change	Increase	Blocked	No change
(i) Stubby	Decrease	Blocked	Decrease	Blocked	No change
Spine volume:					
(i) Mushroom	No change	Decrease	No change	No change	Decrease
(ii) Thin	No change	No change	No change	No change	No change
PSD volume					
(i) Mushroom	No change	No change	No change	No change	Decrease
(ii) Thin	No change	No change	No change	No change	No change
Spinule number					
(i) Mushroom	Increase	Blocked	Increase	Blocked	No change
(ii) Thin	Increase	Blocked	Increase	Blocked	No change
Bouton volume (mushroom spine)	No change	Decreased	Decreased	Decreased	Decreased

Figure legends

Fig. 1A. Categories of dendritic spines and synapses. (A) 3-D reconstruction of a short segment of dendrite with three spine categories distinguished (mushroom, thin, and stubby), and another category – synapses directly on the shaft, shaft synapses. This reconstruction was taken from over 100 serial ultrathin sections, 8 of which are shown in electron micrographs in B (a-h) with individual elements of types of spines and synapses labelled – mushroom spine (a and b); thin spine(c and d); stubby spine (e and f) and shaft synapses (g and h). Abbreviations: (PSD - post synaptic density, sp - spine, den – dendrite; MVB-mutlivesicular body. A schematic of the spine categories is shown in C and can be compared with the reconstructions in A. These categories, as we discuss in the text, most likely represent a continuum where stubby spines become thin and then become thick (mushroom) following neural activation, and *vice versa* when activity/down-regulation occurs. The categories we show are: thin - where the height is usually several times in excess of the width; stubby- where the spine protrudes only slightly from the dendritic shaft; mushroom- where the spine head is large and considerably in excess of the spine neck diameter, shaft synapses are a fourth category where the synapse contacts directly the dendritic shaft (A and C). Scale bar = 1 μm^3 (A); 1 μm (B)

Fig.2. Long-term potentiation (LTP) and long-term depression (LTD) in the middle molecular layer of the dentate gyrus induced by high-frequency stimulation (HFS, arrowhead) of the medial (LTP) or lateral (LTD) perforant paths and blockade by a single i.p. injection of CPP (10 mg/kg, arrow) 3 hours before the tetanization. A minimum of 3 animals were used in each of the groups

A, group mean (± SEM) percent change in two parameters (EPSP slope and spike amplitude) measured on the field potentials during the tests taken before and after HFS on the medial perforant path. Inset (top), examples of field potentials recorded during baseline and 24h after HFS in animals from LTP (left) and CPP groups (right); scale = 2mV, 2msec.

B, averaged change in EPSP slope 30 min and 24h after HFS. As expected, significant potentiation was observed at both time points only for the LTP group. *, $P<0.05$.

C, group mean (± SEM) percent change in two parameters (EPSP slope and spike amplitude) measured on the field potentials during the tests taken before and after HFS on the lateral perforant path. Inset (top), examples of field potentials recorded during baseline and 24h after HFS in animals from LTD (left) and CPP groups (right); scale = 2mV, 2msec.
D, averaged change in EPSP slope 30 min and 24h after HFS. As expected, significant depression was observed at both time points only for the LTD group. *, $P<0.05$

Fig. 3 (A,B) Spinules on mushroom and thin spines. Two electron micrographs (from a series of 100) containing identified synapses and spines. Reconstructions of 100 serial sections of the synapse shown in (A) and (B), and presented in (C). This synaptic contact is on a mushroom spine and 2 spinules from the spine arise as discontinuities in the PSD on the spine head and evaginate into the pre-synaptic bouton. (D, E) 2 serial electron micrographs through a thin spine from which 2 spinules project into an axon, as shown in the 3-D reconstruction in (F). Spinules from thin spine heads are less frequent than on mushroom spines (see below); abbreviations: sp- spine, PSD postsynaptic density.

Fig. 4.A Proportions of each of the categories of synapse on spines after LTP, LTD or CPP treatment. The percentages of each spine type were determined from tissue volumes of up to $800\mu m^3$ in each of 3 animals, in each of the 6 conditions. There is a significant increase in the proportion of mushroom spines (* $p<0.05$), and a decrease in stubby spines (# $p<0.005$) in the LTP group, while LTD causes an increase in thin spines (** $p<0.02$), and a decrease in stubby spines (# $p<0.03$). Both effects are prevented when CPP treatment precedes LTP or LTD induction.
B. The mean number of spinules per spine increases significantly on mushroom spines in the middle molecular layer from 0.2 per spine in control, to 1.4 after induction of LTP (*$p<0.001$), and 0.98 after induction of LTD (* $p<0.001$). In thin spines the number of spinules is smaller per spine (control -0.05) but the increase following LTP or LTD is of similar proportions to mushroom spines (# $p<0.001$). The increases after LTP and LTD were completely blocked by the NMDA antagonist CPP; while CPP alone had no effect on the proportions of the different classes of spines. Data on bars represent standard errors.

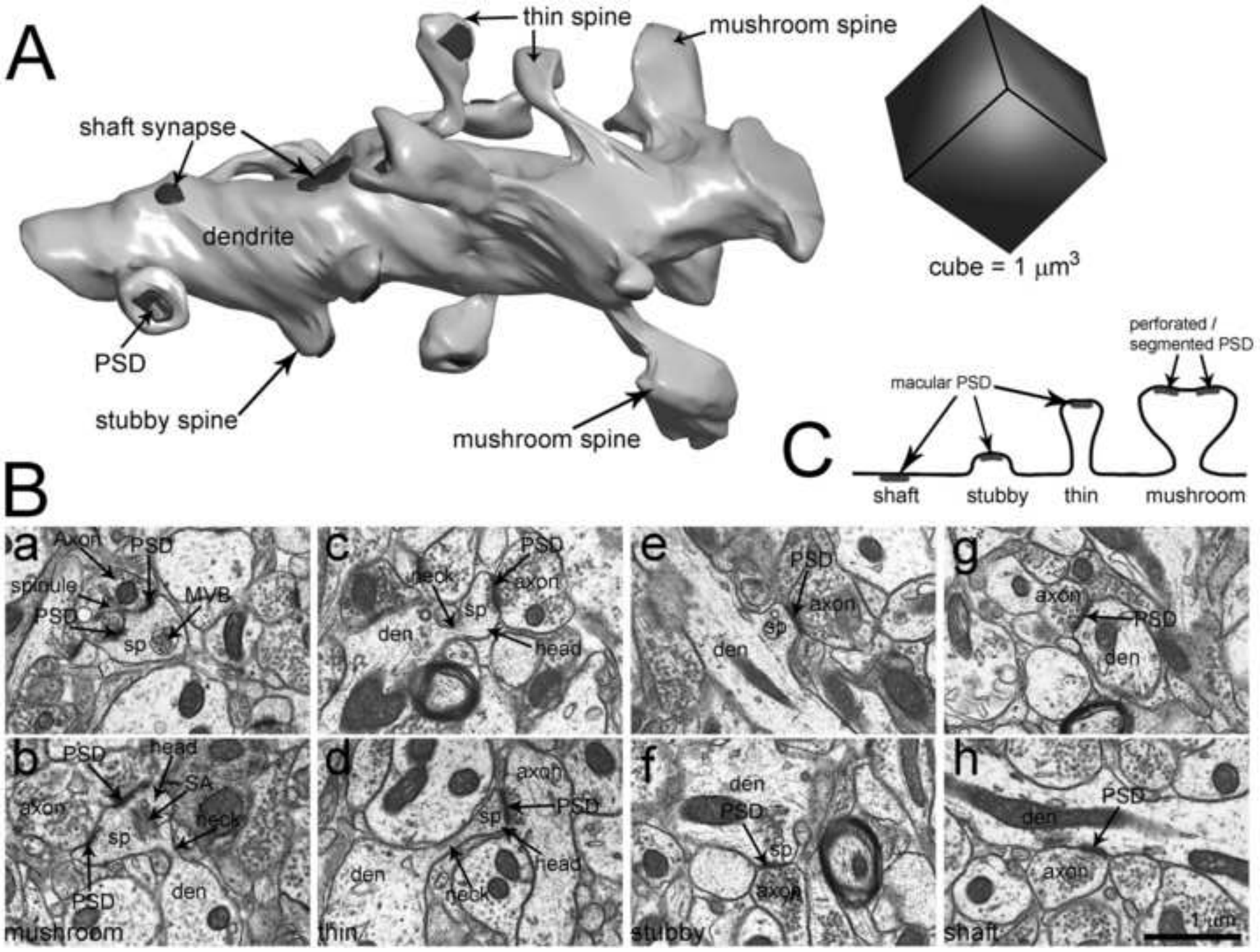
Fig. 5. (A-D) Volume of mushroom and thin spines and volume of PSDs. A minimum of 25 thin mushroom spines, were reconstructed in 3-D per series for each of 3 animals, in each of the 6 conditions to determine volumes.

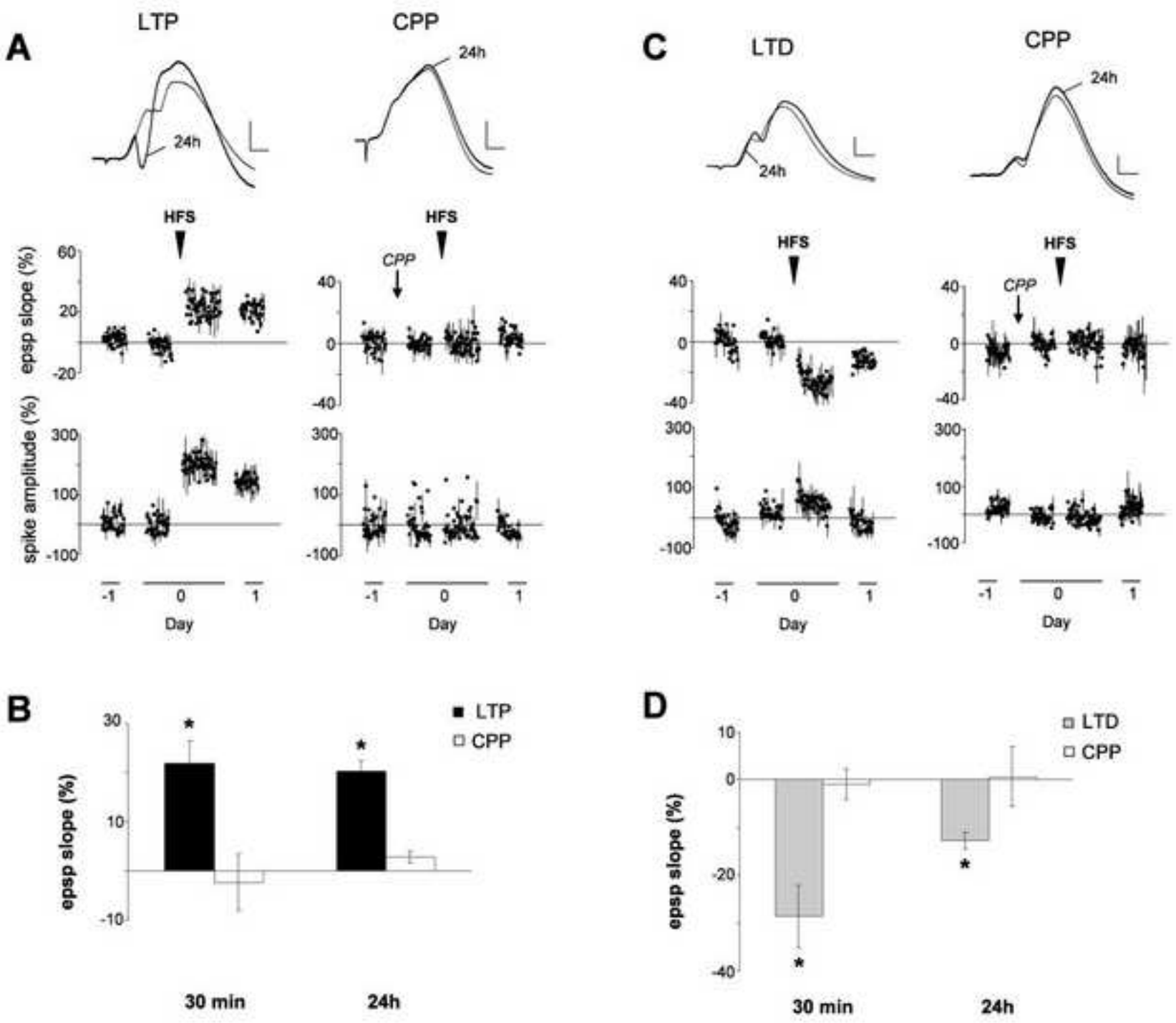
(A, B) The mean volume of mushroom and thin spines did not change significantly after LTP, but there was a significant reduction in mushroom spine volume when induction of LTP was preceded with CPP treatment (*$p<0.04$), as there was when rats (n=3 per group) were treated with CPP alone (**$p<0.01$).

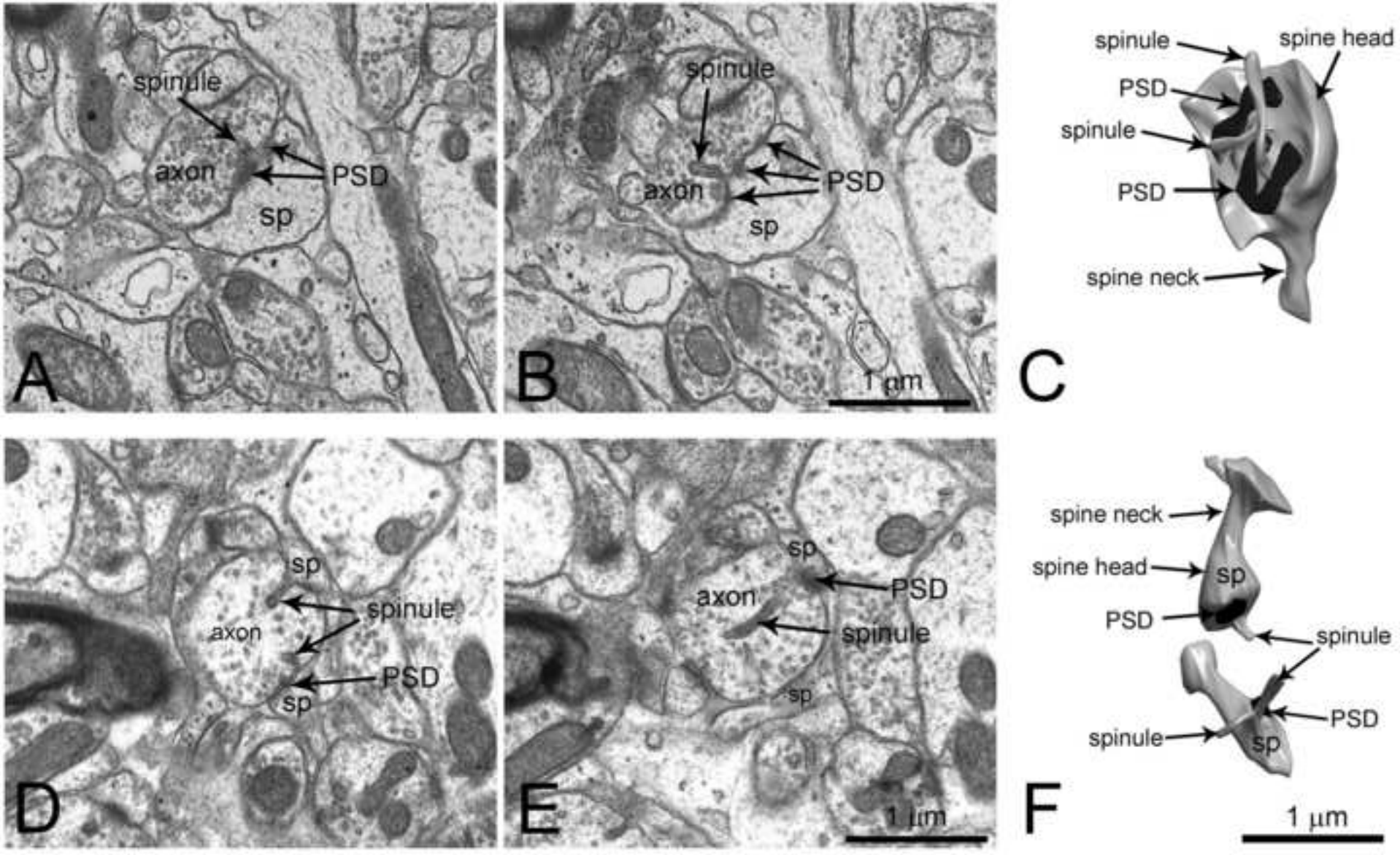
(C) The volume of the PSDs of mushroom spines was unaffected by LTP but decreased significantly (* $p<0.002$) in CPP treated rats. When CPP treatment preceded LTP or LTD treatment there was no significant change in PSD

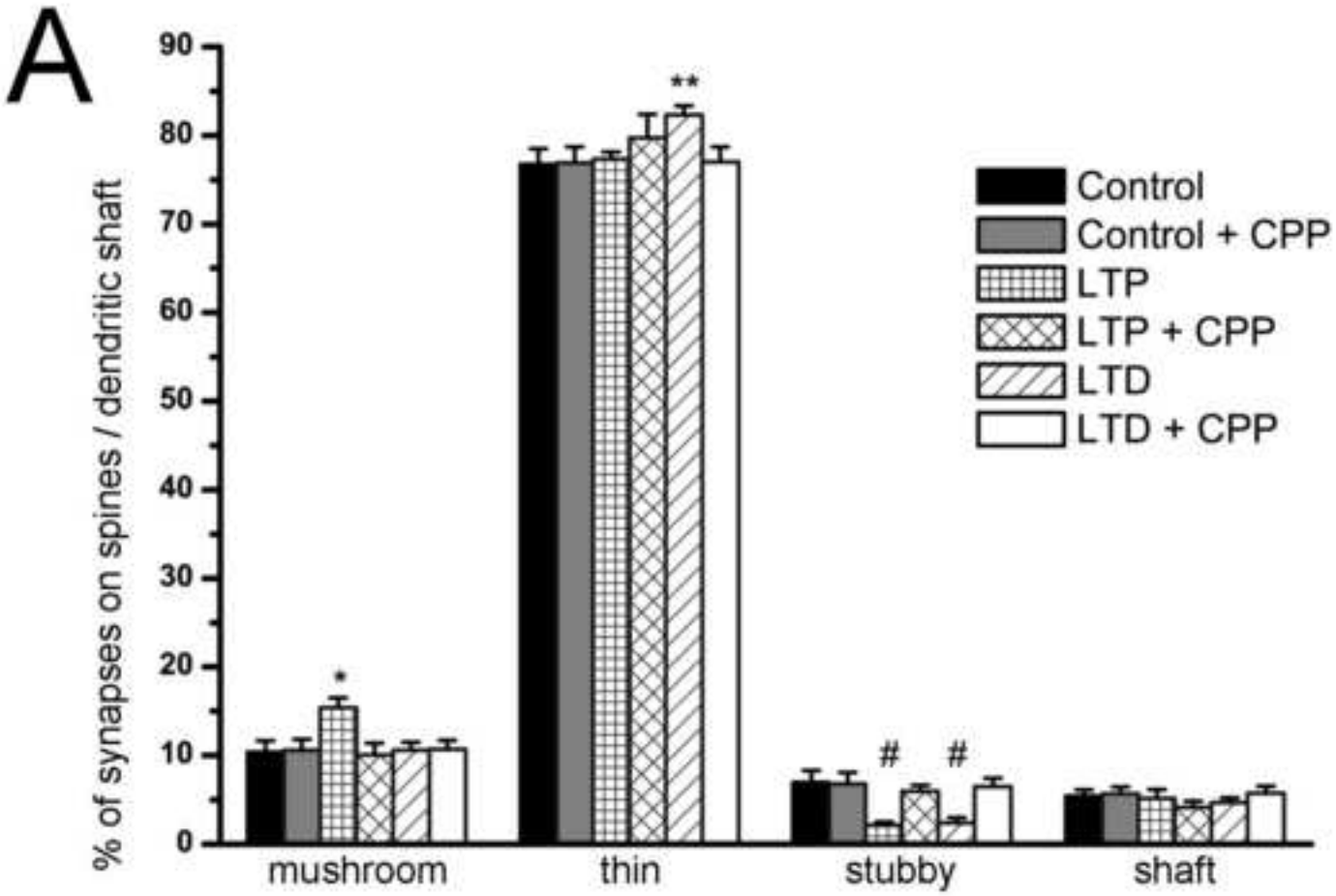
(D). The volume of the PSDs of thin spines was not significantly affected by either LTP, LTD or CPP treatment. Contr: Control

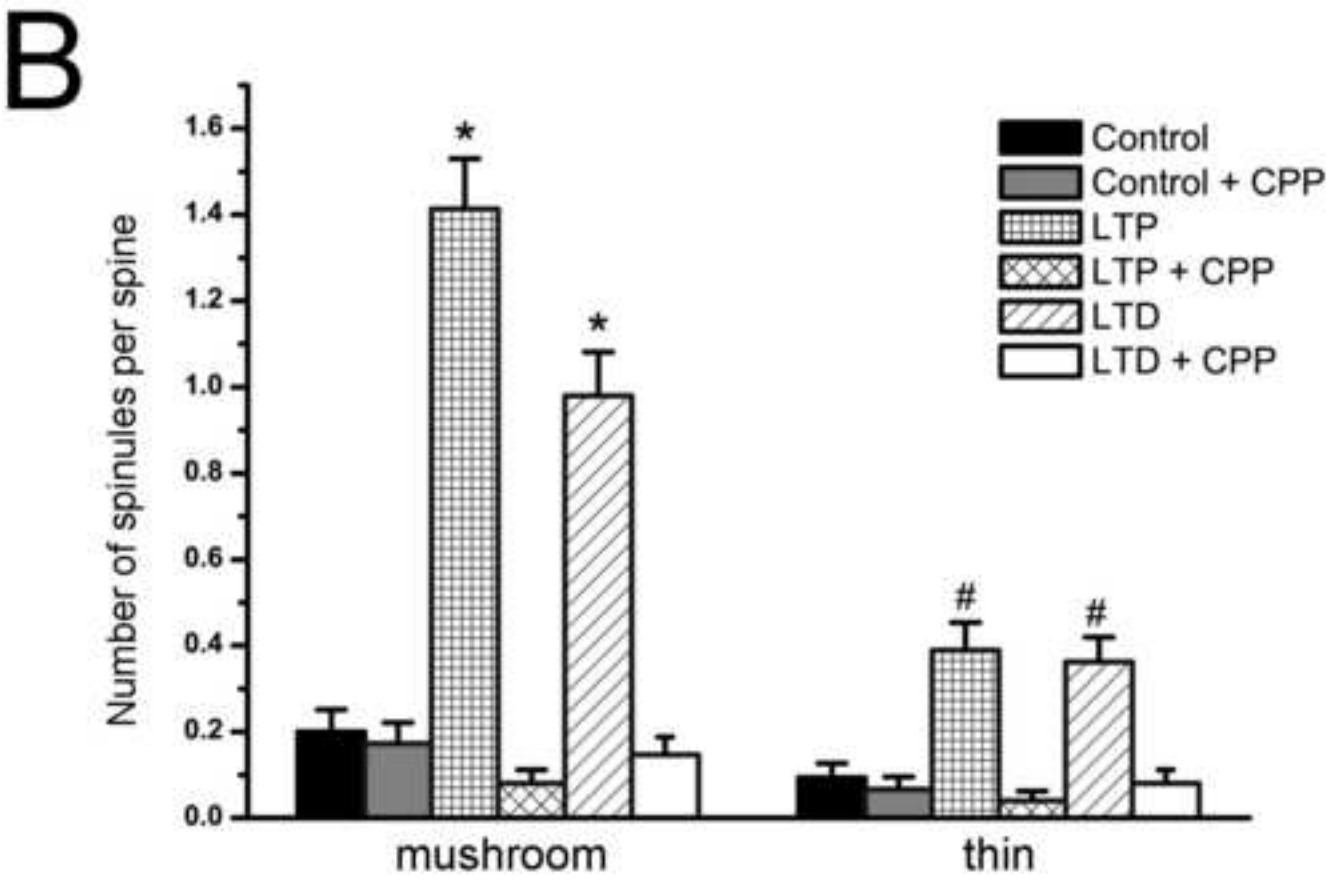
Fig. 6A (Left): an example of a reconstructed presynaptic bouton contacting 2 mushroom spines (sp). Spinules protrude from each spine through the PSD (post synaptic density); Figure 6 A (Right) shows the presynaptic bouton alone with spines and PSD removed. Bouton volume was calculated from the area measurements of the 2-D sections which were used for the 3D reconstruction, scale bar = 1μm. Figure 6B: data for volume changes of presynaptic boutons, a minimum of 25 boutons on mushroom spines were reconstructed in 3-D per series from each of 3 animals, in each of the 6 conditions. The volume of presynaptic boutons was unaffected by LTP but decreases significantly from ~0.6 μm^3 in the control group to ~0.34 μm^3 in the CPP group and was similarly reduced in the LTD group. CPP alone also decreased bouton volume. *values of p between both control and LTP, and the other groups (contr +CPP, LTP+CPP, LTD and LTD+CPP) are <0.05; Contr: Control.











Figure

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